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RNA silencing in germlines—exquisite collaboration of Argonaute proteins with small RNAs for germline survival

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As the proper development of germlines is vital for species preservation, elaborative, regulatory systems for gene expression must operate in germlines. One such system is RNA silencing, sequence-specific gene silencing mechanisms mediated by small RNAs of 20–30 nucleotides long. Indeed, recent studies have revealed that various types of small RNAs are expressed germline-specifically. To preserve the germlines, they collaborate with Argonaute proteins, the catalytic engines in RNA silencing, to inhibit injurious, parasitic genes, transcriptionally or post-transcriptionally. This chapter summarizes the exquisite collaboration of Argonaute proteins with small RNAs in the RNA silencing mechanisms necessary for germline survival in *Drosophila* and mice.

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Introduction

Gene silencing mediated by small RNAs of 20–30 nucleotides (nt) long is collectively called RNA silencing. The key findings that spawned RNA silencing research, particularly in animals, were the identification of *lin-4* miRNA (micro RNA) as a negative regulator of expression of a heterochronic gene, *lin-14* [1] and the discovery of RNA interference or RNAi [2]. The high conservation of *let-7* among species further accelerated the field [3]. Later, various kinds of endogenous small RNAs, not only well-known miRNAs but also newcomers, such as piRNAs (PIWI-interacting RNAs) and esiRNAs (endogenous siRNAs), were identified in organisms ranging from yeast and algae to humans [4–7].

Small RNAs function in RNA silencing by forming a complex with the Argonaute proteins. In the complex, the Argonaute proteins and small RNAs play different roles; small RNAs guide the Argonaute proteins to their targets, while the Argonaute proteins exert enzymatic activities for inhibiting gene expression, mostly by cleaving transcripts or by blocking protein synthesis [4–7]. In this review, we focus on PIWI-interacting RNAs (piRNAs), germline-specific small RNAs in *Drosophila* and mice. As the name indicates, piRNAs are known to be associated with the PIWI proteins, which are germline specific Argonaute proteins. The main issues focused on in this review include the mechanisms of piRNA biogenesis and the mode of action of RNA silencing mediated by the PIWI-piRNA complex. A relatively newly identified small RNA, esiRNA, also appears in this review, because the outcome of esiRNA function, to some extent, overlaps with that of piRNA function.

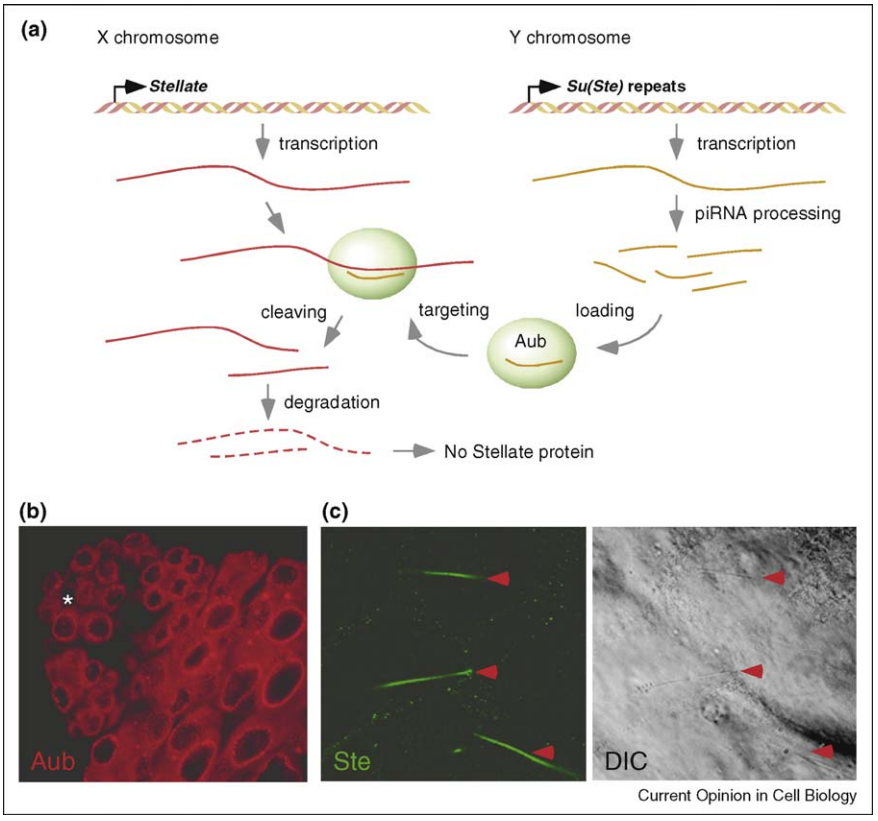
Silencing in *Drosophila* germlines

Earlier studies showed that the *piwi* (P element-induced wimpy testes) gene in *Drosophila* is an essential factor in germline stem cell (GSC) self-renewal [8]. Further investigation demonstrated that mutations in *piwi* cause hyperactivation of retrotransposons and impact on their mobility [9]. The involvement of *piwi* in controlling retrotransposon activity has since become obvious. Identification of small RNAs associated with Piwi in ovaries revealed that Piwi is specifically loaded with a subset of small RNAs [10,11•], originally termed as rasiRNAs (repeat-associated small-interfering RNAs) of 24–30 nt long [12]. rasiRNAs were recently renamed as piRNAs, because the PIWI orthologs in other organisms, such as mice and fish, also associate with the same sort of small RNAs [13]. piRNAs associated with Piwi in *Drosophila* ovaries are mainly derived from intergenic repetitive sequences including transposons in the genome [10,11•]. An *in vitro* assay system showed that Piwi exhibits slicer activity [10]. These results implied that Piwi may function in silencing through a mechanism analogous to RNAi by cleaving gene transcripts that base-pair with a piRNA loaded onto Piwi. However, Piwi is localized in the nucleus [8,10,11•]. Furthermore, recent studies have shown that Piwi forms a complex with HP1, Heterochromatin protein 1, which is involved in heterochromatinization [14]. Thus, Piwi may also be involved in gene silencing at the transcriptional level. Intriguingly, the Piwi-HP1 complex was shown to induce gene 'activation' rather than 'silencing' [14]. In what circumstance Piwi triggers gene activation and what mechanisms control the bidirectionality of Piwi function still remain elusive.

Another member of the PIWI family, *aubergine* (*aub*), was shown to be necessary for pole cell formation [15] and has an impact on the mobility of transposons [16]. Later, it was revealed that Aub in ovaries is associated with piRNAs [11•,17•,18], as is Piwi [10,11•]; the link between Aub and transposon silencing was thus strengthened. Small RNAs associated with Aub in testes were also examined. Needle-shaped aggregation of the Stellate protein in testes causes spermatogenic defects [19]. Genetic studies revealed the involvement of *su(ste)* (*suppressor of stellate*) in *stellate* silencing. Non-coding *su(ste)* shows a strong homology to *stellate* at the nucleotide sequence level and is located as repetitive genes on the Y chromosome. However, the molecular mechanism of *stellate* silencing remained obscure. Later, biochemical analyses showed that piRNAs originating from the *su(ste)* repeats are specifically loaded onto Aub and that the Aub-piRNA complex immunoisolated from testes is able to cleave the *stellate* transcript [18,20]. A direct link between *stellate* silencing and *su(ste)* repeats was uncovered (Figure 1). piRNAs associated with Aub in testes also showed strong complementarities to *vasa*, a factor necessary for germline development [18]. Indeed, in *aub* mutant testes, VASA expression is upregulated. These findings demonstrated that piRNAs might also function in silencing protein-coding genes.

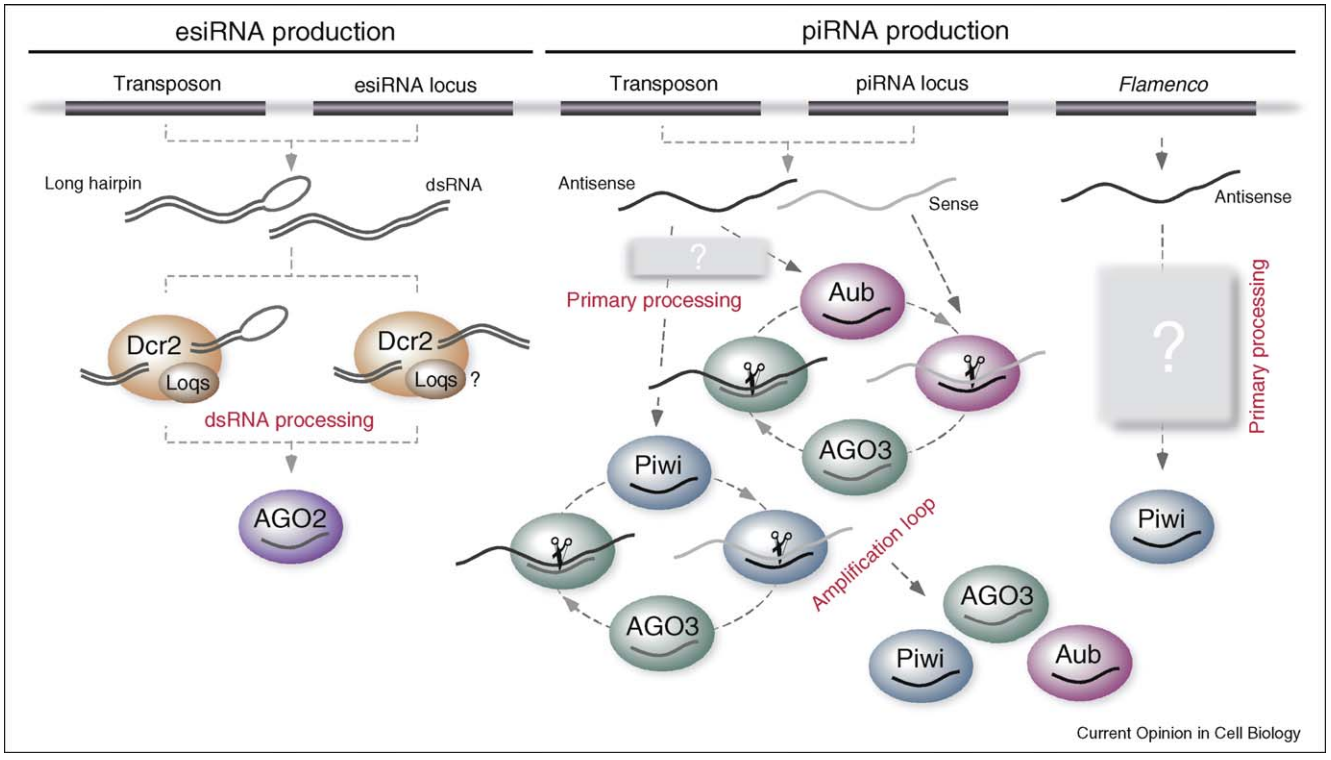
Sequencing analysis of piRNAs in ovaries revealed that piRNAs loaded onto Piwi and Aub are mainly derived from the antisense transcripts of retrotransposons, whereas AGO3, the third and the last member of the PIWI family, preferably binds piRNAs from the sense strand. Moreover, Piwi-piRNAs and Aub-piRNAs show a strong preference for uracil at the 5′ ends (1st-U), while AGO3-piRNAs show a bias for adenine at the 10th nucleotide from the 5′ ends (10th-A) [11•,17•]. *Drosophila* piRNAs are produced in a Dicer-independent manner [20] and all the PIWI proteins showed slicer activity [10,17•,18]. Supported by these data, a piRNA biogenesis model, the amplification loop pathway (*a.k.a.* the Ping-Pong pathway) was proposed [11•,17•] (Figure 2). Other genes, including *Armitage*, *Spindle-E*, *Maelstrom* (*Mael*), *Krimper*, *Squash*, and *Zucchini*, might also be involved in piRNA biogenesis [20–22]. However, their functions in piRNA biogenesis still remain unclear.

Figure 1



Aub function in *Stellate* silencing in *Drosophila* testes. **(a)** A model for *Stellate* silencing mediated by Aub associated with *Su(Ste)*-originating piRNAs. Factors necessary for *Su(Ste)*-originating piRNAs remains unknown. **(b)** Immunofluorescent image of *Drosophila* wild-type testis stained with anti-Aub [17•]. The white asterisk shows where hub, a somatic cell cluster functioning as niche, is located. **(c)** Immunofluorescent (anti-Stellate) and DIC images of *aub* mutant testis. Needle-shaped aggregation of the Stellate protein is indicated by red arrowheads.

Figure 2

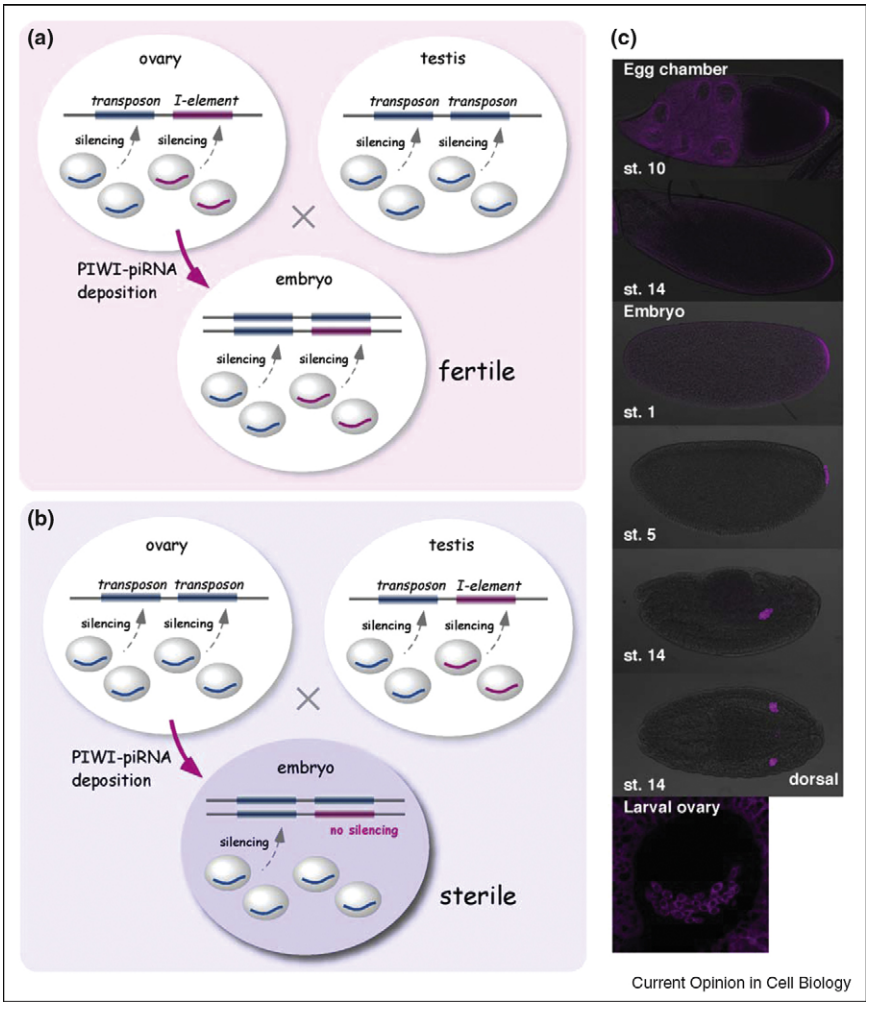


Biogenesis pathways for piRNAs and esiRNAs in *Drosophila*. The primary antisense transcripts of transposons and piRNA loci (shown in black) are processed to piRNAs by unknown mechanisms (called the primary processing), which are loaded onto Aub and Piwi. Then, Aub and Piwi, together with AGO3, consist a piRNA production system, the amplification loop, by utilizing their slicer activity to determine the 5′ end of piRNAs. It is noted that AGO3 is predominantly associated with sense piRNAs (shown in light gray). The resultant RNP complexes of the PIWI family with piRNAs function in silencing transposons. piRNAs originating from a locus on the X chromosome, *flam*, are specifically loaded onto Piwi, meaning that *flam*-piRNAs are only produced by the primary processing pathway. esiRNAs are processed from transcripts of transposons and esiRNA loci. Dicer2 is involved in esiRNA biogenesis. Loqs seems to be also involved in the esiRNA processing, but only when long hairpin-shaped transcripts serve as the substrates. After maturation, esiRNAs are specifically loaded onto AGO2. It is noted that esiRNAs loaded onto AGO2 do not show a strand bias in most cases.

Determination of piRNA origins revealed that a particular locus on the X chromosome, *flamenco* (*flam*), is a hotspot of piRNA production, or is a piRNA cluster [11•]. *flam* was originally identified as a suppressor of specific retrotransposons, *gypsy*, *ZAM*, and *Idefix* [23]. Interestingly, piRNAs originating from *flam* (*flam*-piRNAs) are exclusively loaded onto Piwi [11•]. If *flam*-piRNAs arise by the amplification loop system, *flam*-piRNAs in opposite direction should be detected as AGO3-binders. However, such piRNAs were not detected. Thus another system for piRNA biogenesis, distinct from the amplification loop pathway, must operate [11•]. Currently, this system is called the primary processing pathway [5] (Figure 2). Piwi is expressed in ovarian somas, from which Aub and AGO3 are mostly absent [10,11•,17•,18,24]. It is most likely that in ovarian somas, only the primary processing system produces piRNAs.

Recently, it was shown that piRNAs serve as vectors for epigenetic information in *Drosophila* [25••]. This finding is related to a phenotype called 'hybrid dysgenesis'. The phenotype, accompanied by progeny sterility, is observed when fly strains containing a different set of transposons are crossed. Interestingly, progeny sterility appears only when male-specific transposons are inherited. Why are female-specific transposons tolerated? Recently, Brennecke *et al*. showed that it might be explained by piRNA inheritance [25••] (Figure 3). In their study, the populations of piRNAs in mothers and daughters in normal and dysgenic crosses were extensively examined, which revealed that piRNAs are maternally inherited and that their contents persist from fertilization to adulthood. Maternal piRNAs were thought to be associated with the PIWI proteins because their expression, at least of Aub and Piwi, persists from oocytes to embryos [25••] (Figure 3). Even in testes, piRNAs are produced and associated with the PIWI proteins; however, it seems that the complexes are not inherited and that this lack of paternal deposition is apparently the main cause of hybrid dysgenesis. It should be noted that the age of the mother and/or environmental temperature affects progeny sterility in a dysgenic cross [25••]. Thus lack of paternal

Figure 3



piRNAs serve as vectors for epigenetic information. A phenomenon, called hybrid dysgenesis, is observed when fly strains containing a different set of transposons are crossed. Progeny is fertile when transposons (like I-element) possessed only by a parent are maternally inherited **(a)**. However, when such transposons are paternally inherited, progeny shows sterility **(b)**. Lack of paternal deposition of the PIWI-piRNA complexes is apparently the main cause of hybrid dysgenesis. **(c)** Immunostaining images of *Drosophila* oocytes, embryos, and larval ovary using anti-Aub [17•], which indicate that Aub expression persists from oocytes to embryos.

piRNA inheritance is not the only cause of hybrid dysgenesis.

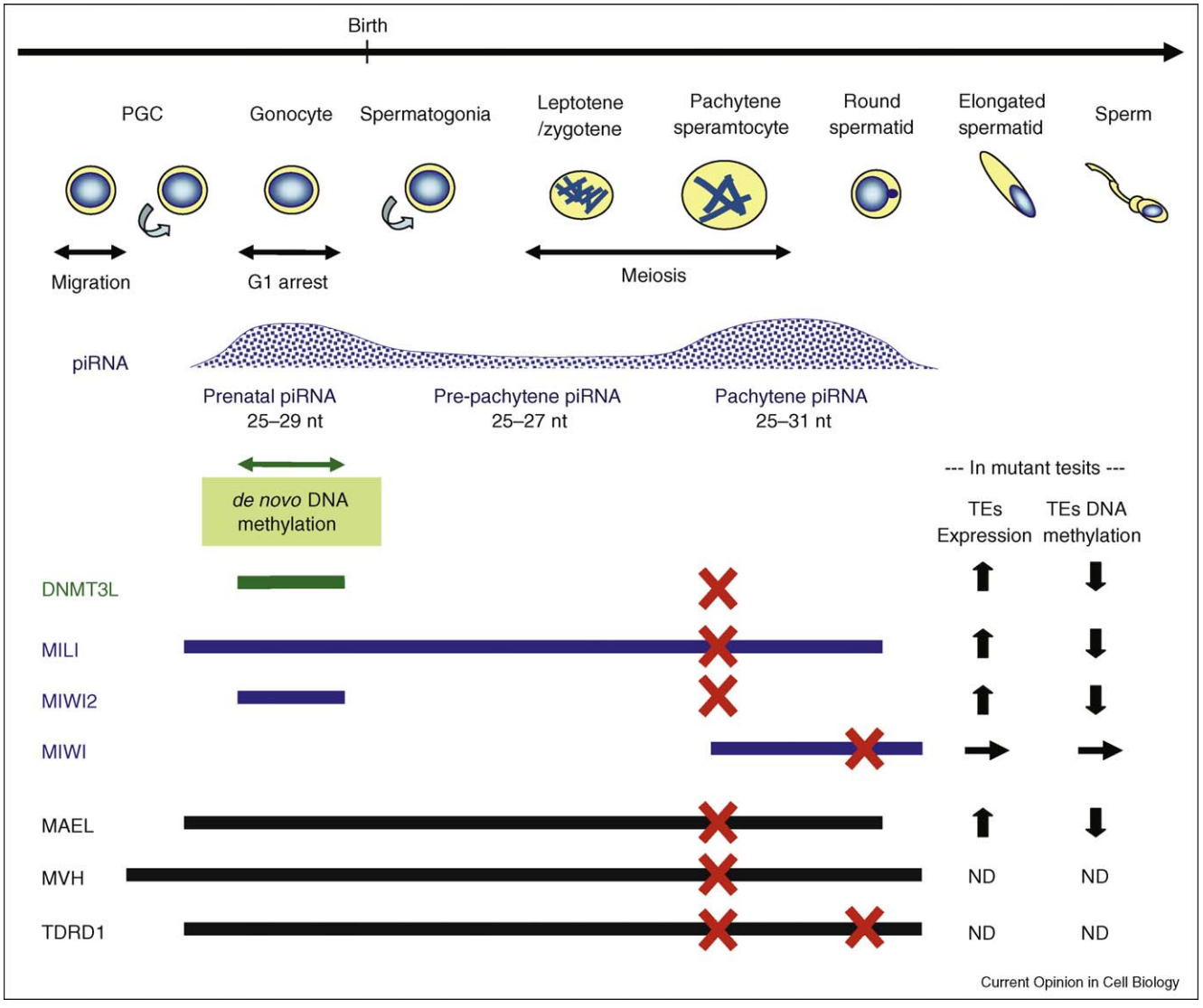
Recent studies revealed that AGO2, a ubiquitously expressed Argonaute, is associated with esiRNAs [26•,27•,28•,29•,30•]. esiRNAs originate from transposons and other intergenic repetitive regions in the genome, as in the case of piRNAs. Some esiRNA sequences may overlap with those of piRNAs. However, esiRNAs and piRNAs are clearly distinct; piRNAs are 24–30 nt long, associate with the PIWI proteins, and are specifically expressed in germlines, whereas esiRNAs are ~21 nt, loaded onto AGO2, and are ubiquitous. Their biogenesis pathways are also distinct; piRNAs are derived from single-stranded RNA precursors through PIWI-slicer activities, whereas esiRNAs are produced from dsRNAs in a Dicer2-dependent manner. Lack of esiRNA production and accumulation causes de-repression of transposons. Thus it is obvious that esiRNAs function in transposon silencing as do piRNAs. Some esiRNAs were also shown to be involved in silencing protein-coding genes.

Silencing in mammalian germlines

In mice, IAP (intercisternal A-particle) and LINE1 (long interspersed nucleotide element) are representative LTRs (long terminal repeats) and non-LTR retrotransposons, respectively. IAP and LINE1 retrotransposons were thought to be maintained in a transcriptionally silent mode by DNA methylation. Indeed, recent studies have

shown that this was the case. Germline-specific gene-knockout studies indicated that DNA methyltransferase 3 (DNMT3) family plays a central role in the *de novo* methylation process [31,34,35]. In fact, it is known that the transposable DNA elements that are demethylated in E10.5-E12.5 PGCs (primordial germ cells) are *de novo* methylated (i.e. reacquisition of DNA methylation) in gonocyte (prospermatogonium) in the E15.5-18.5 fetal testis or in primary oocyte after birth [32]. Two members of the DNMT3 family, DNMT3a2 and DNMT3L, are expressed in gonocytes at E14-18 [33] in male germ cells. Although DNMT3L has no methyltransferase activity, it forms a complex with DNMT3a and is essential for *de novo* DNA methylation. Indeed, *Dnmt3L*-deficient mice fail to establish global *de novo* DNA methylation of transposons in their germ cells (Figure 4). Consequently, transposon expression is enhanced, and the meiosis fails in the mutants [34,35]. Thus *de novo* DNA methylation is

Figure 4



Transposon silencing in mouse spermatogenesis. Upper part: During the spermatogenesis, regulatory regions of the transposable DNA elements are demethylated in PGCs between E10.5 and 11.5. Subsequently, novel DNA methylation (i.e. *de novo* methylation) takes place in the gonocytes (prespermatogonia) in the fetal testes around E15.5-E18.5. piRNAs expressed during spermatogenesis can be divided into three groups, which presumably exhibit differential functions at individual stages. The majority of prenatal piRNAs in fetal gonads correspond to transposons, which implies their functions in *de novo* DNA methylation of transposon genes. Pre-pachytene piRNAs include both transposon-derived and non-transposon-derived piRNAs. Pachytene piRNAs are mostly derived from specific piRNA loci. Prenatal piRNAs are bound with MILI and MIWI2, whereas pre-pachytene piRNAs are associated only with MILI. Pachytene piRNAs are loaded onto MILI and MIWI. Lower part: Horizontal thick lines show the expression periods of genes indicated (DNMT3L, MILI, MIWI2, MIWI, MAEL, MVH, and TDRD1) during spermatogenesis. Red crosses show the time points when loss of individual genes shows phenotypic abnormality. How transposon expression and DNA mathylation are affected when individual genes are abrogated in their mutant mice is summarized on the right-hand side. ND: non-determined.

essential for suppressing transposons and for spermatogenesis in mice.

Three *Piwi* genes are found in the mouse genome: *Miwi* (mouse *Piwi* or *Piwil1*), *Mili* (*Miwi-like* or *Piwil2*), and *Miwi2* (or *Piwil4*). Although all are expressed in germ lineage cells, their expression patterns are different during germ cell differentiation (Figure 4). MILI is expressed from PGCs at E12.5 to round spermatids [36,54], while MIWI2 is mostly expressed in embryonic gonocytes from E15.5; and its expression is diminished rapidly after birth [37•,38••]. MIWI is expressed at the later stage from pachytene spermatocytes to round spermatids in adult testis [39,40]. Loss of *Mili* and *Miwi2* activates retrotransposon gene expression by impairing *de novo* DNA methylation and induces meiotic failure at the pachytene stage just like *Dnmt3L* mutant mice [37•,41]. Thus *Mili* and *Miwi2* are essential for *de novo* DNA methylation of transposons in spermatogenesis. In this regard, *Drosophila* and mouse PIWI proteins are functionally different; because it is unlikely that DNA methylation plays any significant role in gene silencing in flies [42].

As in *Drosophila*, the PIWI proteins in mice, MIWI, MILI, and MIWI2, are associated with piRNAs. Their size distribution differs from one another [37•,43•,44,45] (Figure 4). MIWI is bound with 29–31 nt piRNAs, whereas MILI binds with 24–28 nt piRNAs. MIWI2 associates with 27–29 nt piRNAs. piRNAs expressed in adult testes, pachytene piRNAs, are mostly derived from specific loci in the genome and only 12–17% corresponds to transposons [44–47]. The functions of pachytene piRNAs in spermatogenesis remain unclear. piRNAs in the fetal prenatal stage, prenatal piRNAs, and in neonate stage, prepachytene piRNAs, are mostly derived from transposons [37•,38••,43•]. The expression of prenatal piRNAs is impaired in *Mili* and *Miwi2*-deficient fetal gonocytes, indicating that MILI and MIWI2 are involved in piRNA biogenesis. Considering the DNA methylation defect found in *Mili* and *Miwi2* mutant testes, PIWI and piRNAs probably participate in *de novo* DNA methylation of transposons.

Sequence analysis of piRNAs associated with MILI and MIWI2 revealed that the piRNA production systems, both the primary processing and the amplification loop pathways, also exist in mice. However, there are clear differences between *Drosophila* and mice [38••]. In *Drosophila*, primary piRNAs mainly correspond to the antisense strand of transposons. However, in mice, primary piRNAs are largely processed from mRNAs of active transposons, resulting in production of sense piRNAs. In E12.5 PGC, the regulatory regions in transposons are demethylated. As a result, transposons are expressed, and concomitantly primary sense piRNAs may be processed from their transcripts and loaded onto MILI. In gonocytes, where MIWI2 expression occurs, both MILI and MIWI2 enter the amplification cycle for piRNA production. piRNAs associated with MILI and MIWI2 show a weak but clear strand bias. MILI prefers to bind sense piRNAs, whereas MIWI2 binds antisense piRNAs. Thus it is likely that during the piRNA production, MILI remains associated with primary sense piRNAs, while MIWI2 is loaded with secondary antisense piRNAs that are produced from the amplification loop system. After birth, when MIWI2 is no longer expressed, MILI probably continues to operate the cycle alone during spermatogenesis; because MILI, even after birth, is associated with both types of piRNAs, one with 1st-U and the other with 10th-A [43].

Nuage, the germline-specific cytoplasmic structure found in a variety of organisms including mice and *Drosophila*, has been proposed to play an important role in transposon silencing [48]. In early spermatogenesis in mice, nuage is observed in gonocytes, spermatogonia, and pachytene spermatocytes as multiple granules [49]. Mael, a component of nuage, was shown to be crucial for transposon silencing in spermatogenesis [50•]. Loss of *Mael* is associated with de-repression of transposons, accompanied by reduction of DNA methylation (Figure 4). Interestingly, MILI and MIWI2 also exist in nuage. Subcellular localization of these proteins is dynamic [38••,50•], shuttling from nuage to the nucleus. All the mutants of *mili*, *miwi2*, and *mael*, undergo meiotic arrest at the same point in spermatogenesis and are linked to DNA damage of meiotic chromosomes; strongly suggesting their functional relationship. Indeed, Mael interacts with MILI and MIWI [51]; whether or not MIWI2 interacts with Mael is unclear. Mutations in *Mael* did not change the expression level of prepachytene piRNAs [50•]. Mael may function in transposon silencing downstream of piRNA biogenesis.

In round spermatids, nuage is usually observed as one or two cytoplasmic granules close to the nucleus, which are called chromatoid bodies (CB). CB are likely to be involved in RNA storing and processing because CB contain various proteins related to RNA metabolism. Besides containing MIWI, MILI, and Mael, CB contain germ cell specific components, such as mouse VASA homolog/DEAD box polypeptide 4 (MVH/Ddx4), Tudor domain containing 1/ mouse Tudor repeat 1 (TDRD1/MTR1), and the kinesin motor protein KIF17b, and components of both the miRNA pathway (such as Argonaute proteins, Dicer and GW182) and the RNA-decay pathway (such as the decapping enzyme DCP1a) [52]. However, the roles of these components in CB are largely unknown. In *Miwi*-deficient testis, spermatogenesis is arrested in round spermatids and CB are disrupted [39,53]; thus, MIWI is important for maintaining CB in round spermatids. In contrast to *Mili* and *Miwi2* mutant testes, transposons are not accumulated in *Miwi* mutant

testis. MIWI and MILI interact with piRNAs and mRNAs in polysomes and also some kinds of RNA–protein complexes in adult testes; thus the MIWI-piRNA and MILI-piRNA complexes may regulate translation and/or stability of mRNAs necessary for spermatogenesis [53,55]. A recent study showed that MILI may also positively regulate translation of target genes in germline stem cells [54] as in the case of Piwi in *Drosophila* germlines [14], although such gene activation mechanisms remain obscure.

esiRNAs are also found in mouse oocyte [56•,57•]. esiRNAs in mice are mapped to retrotrasposons or other genomic regions that produce transcripts, including mRNAs and pseudogene transcripts, which are capable of forming dsRNA structures. Inverted repeat structures, bidirectional transcription at one locus, and sense and antisense transcripts from different loci are the main sources of the dsRNA precursors. In *Dicer* and *Ago2*-deficient oocytes, esiRNA levels are decreased. Expression levels of RLTR10 and MTA retrotransposons and protein-coding transcripts complementary to esiRNAs are increased. Thus esiRNAs from naturally occurring dsRNAs regulate both protein-coding transcripts and some transposons in mouse oocytes. LINE1 expression was not changed in *Dicer*, *Ago2*-deficient oocytes, and *Mili*-deficient oocytes. LINE1 expression may be regulated through the combination of piRNAs and esiRNAs in oocytes by a post-transcriptional gene silencing mechanism, that is, mRNA degradation, whereas IAP expression is regulated only through piRNAs associated with MILI.

Silencing in germline: other species

Silencing in the germline is not restricted in *Drosophila* and mice. Analogous pathways are present in other species, including fish and nematodes. Zebrafish expresses two PIWIs, Ziwi and Zili [5,6]. In zebrafish, piRNAs are expressed in both ovaries and testes, as in *Drosophila* [58]. Identification and characterization of zebrafish piRNAs revealed that they are mainly derived from retrotransposons. Further data suggested that the amplification loop system also exists in zebrafish as well. Both Ziwi and Zili were shown to be necessary for maintaining germlines [58,59].

Deep sequencing analysis in *C. elegans* revealed that this organism expresses small RNAs of 21 nt having 1st-U; they were termed as 21U-RNAs [60]. Their size is very close to that of esiRNAs; however, 21U-RNAs are required for maintaining the gemlines, like piRNAs. The protein partner of 21U-RNAs is PRG-1 (Piwi-related gene-1), a member of the PIWI family in *C. elegans* [61]. Accordingly, 21U-RNAs are thought to be *C. elegans* piRNAs. They fail to exhibit complementarities to other expressed sequences [60,61]; thus their function remains unclear.

Conclusions

Transposons represent a large portion of the genome (in flies and mice, roughly 30% and 40%, respectively [62,63]) and tend to be more active in germlines. This is because many germline-specific genes must be expressed in gonads and thus the chromosomes need to be kept in a looser structure for a period during their development. In such an environment, transposons have a higher innate chance to transpose, which poses a considerable threat to the host species. To restrain transposons, the germline must have acquired powerful additional means to suppress their activities; namely, piRNAs and the PIWI proteins that amplify and associate with piRNAs. In *Drosophila*, AGO2 in ovaries is loaded with esiRNAs and exogenous siRNAs and shows slicer activity *in vitro* [26•,64]. However, AGO2 in fly ovaries seems to remain inactive *in vivo* until oocytes become almost ready to be fertilized [65]. This may be an alternative reason, at least in *Drosophila*, for the observation that the PIWI-piRNA system is equipped in germlines for silencing invasive transposable elements.

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- • of special interest
- •• of outstanding interest

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